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ATTN: MCMR-CDR-P
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

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US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ-I
3100 Ricketts Point Road
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RESEARCH ARTICLE

Evaluation of nine oximes on in vivo reactivation of blood, brain, and tissue cholinesterase activity inhibited by organophosphorus nerve agents at lethal dose

Tsung-Ming Shih, Jacob W. Skovira, John C. O'Donnell, and John H. McDonough

Pharmacology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400, USA

Abstract

The capability of several oximes (HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD 585, ICD 692, ICD 3805, and 2-PAM) to reactivate in vivo AChE inhibited by the nerve agents sarin, cyclosarin, VX, or VR in blood, brain regions, and peripheral tissues in guinea pigs was examined and compared. Animals were injected subcutaneously with 1.0 LD_{50} of sarin, cyclosarin, VR, or VX, and treated intramuscularly 5 min later with one of these compounds. Toxic signs and lethality were monitored, and tissue AChE activities were determined at 60 min after nerve agent. The animals exposed to sarin or cyclosarin, alone or with non-oxime treatment, some died within 60 min; however, when treated with an oxime, no animal died. For VR or VX, all animals survived for 60 min after exposure, with or without non-oxime or oxime therapy. These nerve agents caused differential degrees of inhibition: in whole blood sarin = cyclosarin > VR = VX; in brain regions sarin > cyclosarin > VX > VR; and in peripheral tissues sarin > VX > cyclosarin > VR. These oximes exhibited differential potency in reactivating nerve agent-inhibited AChE in various peripheral tissues, but not AChE activity in the brain regions. There was no difference in the AChE reactivating potency between the dichloride and dimethanesulfonate salts of HI-6. AChE inhibited by sarin was the most and cyclosarin the least susceptible to oxime reactivation. Overall, MMB-4 appeared to be, among all oximes tested, the most effective in vivo AChE reactivator against the broadest spectrum of nerve agents.

Keywords: *Acetylcholinesterase; cholinesterase inhibitors; cholinesterase reactivation; cyclosarin; guinea pig; HI-6; HLö7; methoxime; nerve agents; organophosphorus compounds; oximes; pralidoxime; sarin; trimedoxime; VR; VX*

Abbreviations: Ach, acetylcholine; AChE, acetylcholinesterase; BCA, bicinchoninic acid; ChE, cholinesterase; DiCl, dichloride; DMS, dimethanesulfonate; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; GB, sarin; GF, cyclosarin; HI-6, 1-(4-carbamoylpyridino) methoxymethyl-2-(hydroxyiminomethyl) pyridinium; im, intramuscular; ip, intraperitoneal; LD_{50}, median lethal dose; MMB-4, methoxime; OP, organophosphorus compound; 2-PAM, pyridine-2-aldoxime methylchloride; PB, pyridostigmine bromide; RBC, red blood cell; sc, subcutaneous; TMB-4, trimedoxime; WB, whole blood

Introduction

The potential for exposure to organophosphorus (OP) nerve agents exists on the battlefield and as a terrorist threat to civilian populations. These agents are extremely potent inhibitors of the cholinesterase (ChE) enzymes. Their toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in particular, acetylcholinesterase (AChE), and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (Moore et al., 1995; Taylor, 2001; Aas, 2003). In the event of nerve agent exposure, immediate therapeutic treatment with an anti-cholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites, and an oxime, such as 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), P2S (N-methylpyridinium-2-aldoxime methansulfonate), obidoxime (Toxogonin®) or HI-6 (1-(((4-(aminocarbonyl) pyridinio)methoxy) methyl)-2-((hydroxyimino)methyl) pyridinium), is used to reactivate any unaged inhibited enzyme (Moore et al., 1995; Aas, 2003).

Oximes refer to compounds comprised of an oxime moiety (R-CH=NOH) attached to a quaternary nitrogen

Address for Correspondence: Tsung-Ming Shih, Commander, US Army Medical Research Institute of Chemical Defense, ATTN: MCMR-CDR-P (Dr. T.-M. Shih), 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA. Tel: 410-436-3414. Fax: 410-436-2072. E-mail: tsungming.a.shih@us.army.mil

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pyridinium ring. They reactivate OP-inhibited AChE by dephosphonylating the enzyme's active site by interacting with a nearby anionic subsite. Reactivation occurs through nucleophilic attack by the oxime on the phosphorous atom, splitting an oxime-phosphonate away from the active site. The regenerated esteratic site is subsequently able to bind and cleave its normal substrate, ACh. This AChE reactivating action of the oximes is considered to be the major mechanism of their antidotal action in reversing the toxic/lethal effects of nerve agents (Maxwell et al., 2006). Several oximes are potentially beneficial for the antidotal treatment of OP nerve agent intoxication (Dawson, 1994; Kassa, 1998; 2002; Aas, 2003). The basic structures are very similar, differing only by the number of pyridinium rings and by the position of the oxime moiety on the ring (see Figure 1).

2-PAM is a mono-pyridinium oxime currently used in the US for the emergency treatment of OP nerve agent exposure. Some countries use different salts (e.g. methanesulfonate, P2S) of 2-PAM (Dawson, 1994). Other countries prefer the bis-pyridinium obidoxime (Toxogonin®). Recently some countries have used HI-6 dichloride as a bis-pyridinium oxime antidote for OP compounds (Moore et al., 1995; Aas, 2003).

Although 2-PAM provides adequate protection against some nerve agents, such as sarin (GB) and VX, it is less effective against other nerve agents, such as tabun, soman, cyclosarin (GF), and a Russian V-agent (VR) (Boskovic et al., 1984). For this reason, numerous studies in the last several decades have focused on developing improved oxime reactivators. In recent years, several oximes, such as HI-6, HLö7 (ICD 2445), MMB-4 (ICD 039), TMB-4 (trimethoxime), carboxime, ICD 585, ICD 692, and ICD 3805, have been found to possess much better antidotal capacity than 2-PAM does in response to intoxication to OP nerve agents in animal studies (Dawson, 1994; Kassa, 1998; 2002; 2005; Nechiporenko and Zatsepin, 2003; Dishovsky, 2005; Kokshareva et al., 2005; Antonijevic and Stojiljkovic, 2007; Eyer, 2008; I. Koplovitz, unpublished observations). Even though several of these oximes have been studied for their ability to reactivate soman-, GF-, or VX-inhibited AChE activity individually in vivo (Harris and Stitcher, 1983; Lundy and Shih, 1983; Harris et al., 1989, 1990; Shih et al., 1991; Shih, 1993; Koplovitz and Stewart, 1994; Kassa and Cabal, 1999), their broad spectrum capacity to reactivate nerve agent-inhibited AChE activity has not yet been evaluated and compared systematically. The ideal oxime should be able to effectively reactivate AChE inhibited by OP agents of diverse structures.

The dichloride (DiCl) salt of HI-6 was the original salt used by some countries (Moore et al., 1995) and the one we have studied for years (Lundy and Shih, 1983; Shih et al., 1991; Shih, 1993). The dimethanesulfonate (DMS) salt of HI-6 has been found to be more water-soluble than the DiCl salt and, thus, may provide better bioavailability upon intramuscular (im) administration. The DMS salt is the formulation of HI-6 that is proposed to be utilized in a new HI-6 autoinjector, which is in development (Clair et al., 2000). Studies with the DMS salt are needed to investigate its pharmacodynamic in vivo equivalency with the DiCl salt (Krummer et al., 2002).

The objectives of this study were, therefore, to compare several oximes (HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD 585, ICD 692, and ICD 3805) with 2-PAM (see Figure 1) in their ability to reactivate in vivo blood (whole blood and red blood cell) and tissue (brain regions, spinal cord, diaphragm, heart, skeletal muscle) AChE activity inhibited by a variety of OP nerve agents and to compare the AChE-reactivating potency of the two salts of HI-6. Two structurally similar compounds without an oxime group (SAD128 and ICD 4157) were included to serve as negative controls in AChE-reactivation (Figure 1). The overall goals are to generate a database for selecting a broad-spectrum oxime that is significantly more effective than 2-PAM as an antidote for numerous nerve agents and to support the research and development efforts to replace the current oxime 2-PAM in the antidotal regimen (Singh et al., 2007; Saxena et al., 2008).

Materials and methods

Animal welfare

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, by the Institute of Laboratory Animal Resources, National Research Council. The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC) of the US Army Medical Research Institute of Chemical Defense. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Subjects

Male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250–300 g were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature (21 ± 2°C)- and humidity (50 ± 10%)-controlled quarters that were maintained on a 12-h light-dark schedule (with lights on at 06:00 h). Laboratory chow and tap water were freely available whenever the animals were in home cages. Animals were allowed to acclimate for 1 week prior to experimentation.

Materials

Saline (U.S.P.) was purchased from Braun Medical Inc. (Irvin, CA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). AChE from electric eel, bovine serum albumin, and acetylthiocholine iodide were purchased from Sigma-Aldrich (St. Louis, MO). Pyridine-2-aldoxime methylchloride (2-PAM) was purchased from Ayerst Labs, Inc. (New York, NY). SAD128 (1,1'-[oxybis(methylene)]

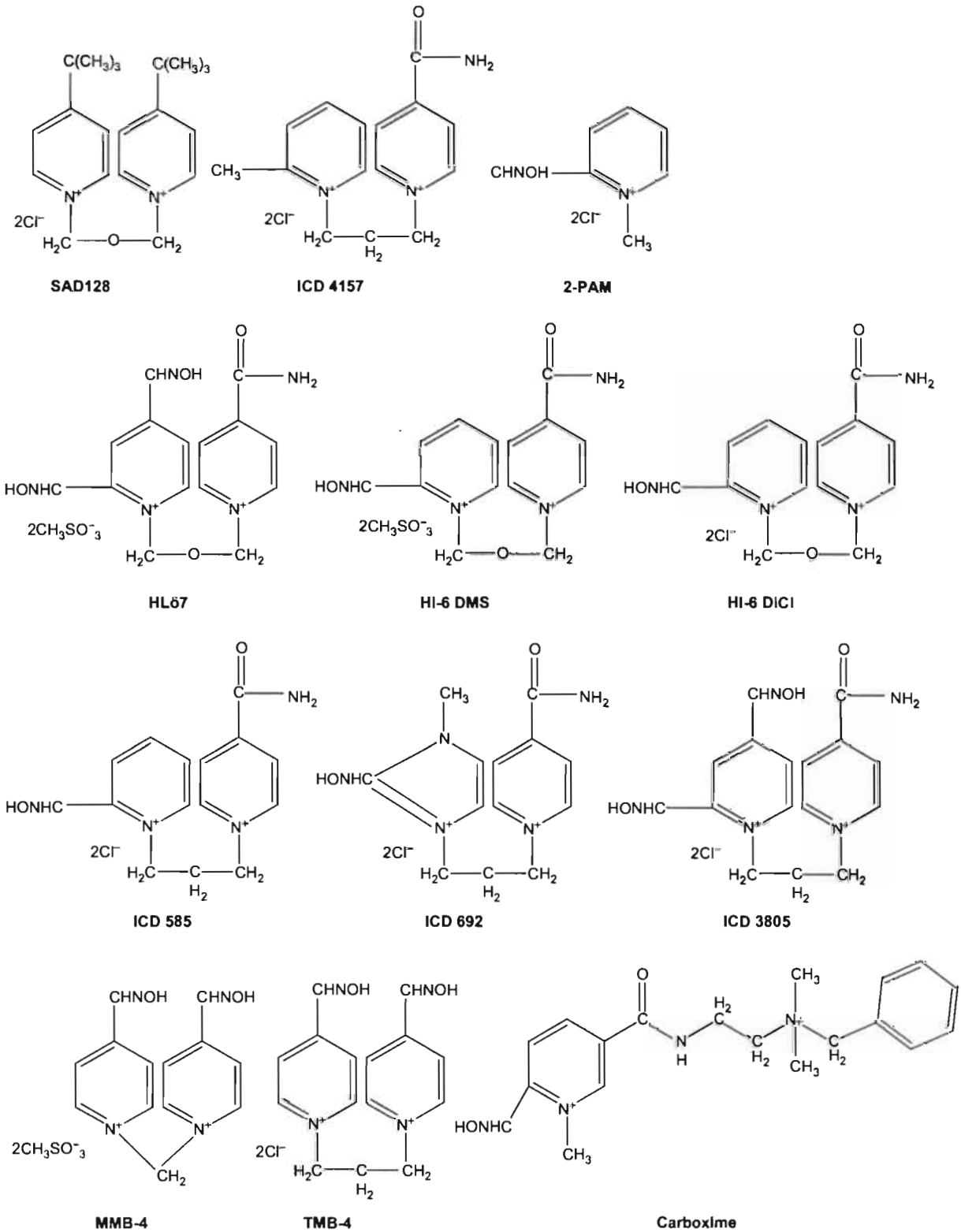

Figure 1. Chemical structures of non-oximes (SAD128 and ICD 4157) and oximes (2-PAM, HLö7 [ICD 2445], HI-6 DMS, HI-6 DiCl, ICD 585, ICD 692, ICD 3805, MMB-4 [ICD 039], TMB-4, and carboxime).

bis[(4-tert-butyl)-pyridinium] dichloride), HLö7 (ICD2445; 1-[[[4-(aminocarbonyl)pyridinio]methoxy] methyl]-2, 4-bis[(hydroxyimino) methyl]pyridinium dimethanesulfonate), MMB-4 (methoxime; ICD 039; 1,1'-methylenebis[4-[(hydroxyimino)methyl]pyridinium] dichloride), HI-6 DiCl (1-(((4-(aminocarbonyl)pyridinio) methoxy)methyl)-2-((hydroxyimino)methyl)pyridinium dichloride), HI-6 DMS, TMB-4 (trimedoxime; 1,1'-trimethyl bis-[4-formyl pyridinium chloride] dioxime), carboxime (1,4-methyl-5-[2'-(benzyldimethylammonium)ethyl]

carbamoylpyridinium-2-aldoxime dichloride), ICD 585 (1-(4-aminocarbonylpyridinio)-3(2-hydroxyiminomethylpyridinio)propane dichloride monohydrate), ICD 692 (1-[1-(2-hydroxyiminomethyl-3-methyl)imidazolo]-3-[4-carbamoylpyridinium]-propane dichloride hydrate), ICD 4157 (1-[3-[4-aminocarbonylpyridinio] propyl]-2-methylpyridinium dichloride hydrate), and ICD 3805 (3-[4-carbamoyl-1-pyridino]-1-[2,4-bis(hydroxyiminomethyl)-1-pyridino]propane dichloride hydrate) were obtained from the depository at the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Silver Spring, MD). The purity of all oximes was >99%, as determined by elemental and HPLC analyses. Bicinchoninic acid (BCA) Protein Assay Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1 N sodium hydroxide), BCA Protein Assay Reagent B (4% cupric sulfate), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). DTNB was prepared in Tris buffer (0.05 M, pH 8.2) to a concentration of 0.424 M. Attane™ (Isofluane, USP) was purchased from Minrad, Inc. (Bethlehem, PA). The four OP nerve agents studied were sarin (GB; isopropyl methylphosphonofluoridate), cyclosarin (GF; cyclohexyl methylphosphonofluoridate), VX (0-ethyl S-(2-(diisopropylamino) ethyl) methylphosphonothioate), and a Russian V-type agent designated VR (0-isobutyl S-(2-(diethylamino)ethyl)methylphosphonothioate). They were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). The purity of these nerve agents was >98.5%, as determined by ^{31}P NMR. Nerve agents were diluted in ice-cold saline prior to subcutaneous (sc) injection. All non-oxime and oxime compounds, including 2-PAM, were prepared in saline for intramuscular (im) injection. Injection volume was 0.5 ml/kg for administration of all OP nerve agents and oxime compounds.

Experimental procedure

One-to-three days prior to the experiment, baseline blood (0.5 ml) was drawn using the toenail clip method (Vallejo-Freire, 1951) and was collected into a 1.0-ml microfuge tube containing 50 µl of heparin sodium (15 units/ml) to determine baseline AChE activity in whole blood (WB) and red blood cell (RBC). On the day of the study, groups of guinea pigs were injected subcutaneously with either saline (0.5 ml/kg) or a 1.0 LD_{50} dose of GB (42.0 µg/kg), GF (57.0 µg/kg), VR (11.3 µg/kg), or VX (8.0 µg/kg). Five minutes later, when the inhibition of AChE activity by these nerve agents reached maximum in the blood (Shih et al., 2005), saline (0.5 ml/kg), SAD128 (20.7 mg/kg), ICD 4157 (20.1 mg/kg), MMB-4 (19.1 mg/kg), carboxime (24.0 mg/kg), HLö7 (30.2 mg/kg), HI-6 DMS (27.8 mg/kg), HI-6 DiCl (21.9 mg/kg), 2-PAM (25.0 mg/kg), ICD 585 (21.8 mg/kg), ICD 692 (22.0 mg/kg), ICD 3805 (23.2 mg/kg), or TMB-4 (20 mg/kg) was given intramuscularly. The group of animals that received both sc saline (no nerve agent) and im saline (no oxime) injections served as overall controls (saline/saline group).

The dose of 2-PAM (25 mg/kg, im) was equivalent to 145.0 µmol/kg, im, which is equivalent to three autoinjector doses (as in Mark I Nerve Agent Antidote Kit) in a 70-kg person. The dose of TMB-4 was 20 mg/kg (43 µmol/kg; equivalent to its ¼ LD_{50} dose). The dose of other oximes studied was 58.0 µmol/kg, im, which was equivalent to the maximum three autoinjector doses to be given to a 70-kg person (based on HI-6 DiCl) (Clair et al., 2000).

ICD 4157 and SAD128 served as negative controls. ICD 4157 is structurally similar to ICD 585 and SAD128 is structurally similar to HI-6. The dose of ICD 4157 used for this study was 20.1 mg/kg, im (equivalent to 58 µmol/kg), while the dose of SAD128 used was 20.7 mg/kg, im (53.6 µmol/kg; equivalent to its ¼ LD_{50} dose).

Sixty minutes after nerve agent administration, the animals were euthanized following isoflurane anesthesia (5% in oxygen). Trunk blood was collected into a 1.0-ml microfuge tube containing 50 µl of heparin sodium solution (15 units/ml). For the WB sample, 20 µl of collected blood was diluted (1:25) in 1% Triton-X100 (in water) solution. The original blood sample was then centrifuged (5 min at 16,000 × g) and 10 µl of the RBC were then diluted (1:50) in 1% Triton-X100 solution. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum) and peripheral tissues (diaphragm, heart, and skeletal muscle) were dissected. Brain and peripheral samples were diluted 1:20 and 1:5, respectively, in 1% Triton-X100 solution (in water), and then homogenized. Homogenized samples were then spun in a centrifuge at 31,000 × g (brain for 20 min and peripheral tissue for 30 min) and supernatants saved. The processed brain and peripheral tissue supernatants, and RBC and WB samples were kept frozen at -80°C until analysis. The AChE activity was measured spectrophotometrically using a variation of the microplate method modified from Ellman et al. (1961), and a BCA protein assay was used to obtain protein concentrations in the tissue samples to standardized AChE levels among tissues, as was reported elsewhere (Shih et al., 2005).

AChE analysis

On the day of AChE analysis, the brain and peripheral tissue samples were thawed, and three 7-µl replicates of each were pipetted into a 96-well microplate (UV Star, Greiner, Longwood, FL). Three 10-µl replicates of the WB and RBC samples were pipetted into the microplates. Standard curves were established by adding 7 µl (for brain and peripheral tissue samples) or 10 µl (for WB and RBC samples) AChE from electric eel at 3.75, 7.5, and 15 U/ml. Twenty microliters of deionized water was added to each well containing brain and peripheral tissue samples, and 17 µl of deionized water was added to each WB and RBC sample. Following the addition of water, 200 µl of DTNB (0.424 M, pH 8.2) was added as the chromatophore to each sample well. Each microplate was then incubated for 10 min at 37°C before being placed in the Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) where it was allowed to shake for 2 min. Immediately after, 30 µl of the substrate

acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 412 nm (at 20 s intervals) for 3.5 min, and the activity (μmole/ml/min) was determined using Softmax plus 4.3 LS software (Molecular Devices). Special care was taken to avoid any interference of oximes with acetylthiocholine in AChE activity analysis (Sakurada et al., 2006; Worek and Eyer, 2006; Petroianu, 2007).

Protein analysis

Protein levels in the tissue samples were determined by a BCA protein assay method (Pierce Biotechnology, Inc.). The standard curve was created using bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml. Three replicates of 10 μl for each brain tissue sample were added to individual microplate wells. To each well of brain tissue samples 200 μl of working reagent was then added. Three replicates of 5 μl for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 μl of deionized water before adding 200 μl of BCA working reagent. The microplates were shaken for 30 s and then incubated at 37°C for 30 min. The microplates were allowed to cool to room temperature before being read using the Spectramax Plus microplate reader and Softmax Plus 4.3 LS software as described above. After obtaining the protein contents of each tissue sample, the AChE activity is then expressed as μmol substrate hydrolyzed/g protein/min for brain and peripheral tissues.

Data analysis

AChE activity was initially expressed as μmol substrate hydrolysed/ml/min for RBC and WB (Table 1) and then converted to percentage of the individual animal's baseline AChE value that was obtained one to three days prior to experimental study (Figures 2 and 3). In peripheral tissues and brain regions the AChE activity was initially expressed as μmol substrate hydrolyzed/g protein/min (Tables 1 and 2) and then expressed as percentage of the saline-treated control AChE value obtained on the day of experiment (Figures 4–6). The enzymatic activities of the treatment groups were then expressed as percentage of the saline/saline control group (mean ± SEM % of control value) within a nerve agent. Statistical analysis was performed using a one-way ANOVA to compare across tissues for basal AChE activity, among tissues across nerve agents, and across treatment groups for each nerve agent. A post-hoc Tukey test was used for multiple comparisons. Statistical significance is defined as $p < 0.05$. Only comparisons between each treatment group in which AChE activity was significantly higher than the activity in the nerve agent/saline-treated group or the 2-PAM-treated group are discussed.

In this report, the AChE activity reactivated by the specific oxime in any tissue is considered to be that portion of the activity in a nerve agent/oxime-treated group that exceeded the activity in the nerve agent/saline-treated group (at 60 min).

Table 1. AChE activity in peripheral tissues and blood in control and nerve agent-intoxicated guinea pigs.*

	μmol substrate hydrolyzed/g protein/min			μmol substrate hydrolyzed/ml/min	
	Diaphragm	Heart	Skeletal Muscle	RBC	WB
Control AChE activity,† mean ± SEM					
Control	14.32 ± 0.76	18.23 ± 0.56	10.50 ± 0.43	2.18 ± 0.06	2.45 ± 0.06
% of control AChE activity at 60 min,† mean ± SEM					
GB	25.7 ± 2.2	15.0 ± 1.5	34.2 ± 3.6	8.7 ± 1.1	8.8 ± 1.5
GF	35.3 ± 3.3	34.7 ± 2.9	48.3 ± 4.1	5.9 ± 1.1	16.6 ± 2.7
VR	48.1 ± 4.1	54.9 ± 2.0	60.9 ± 4.0	6.6 ± 1.2	30.8 ± 3.6
VX	27.7 ± 1.2	23.6 ± 1.1	32.1 ± 2.4	6.3 ± 1.0	26.8 ± 2.1

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 LD_{50} dose of the nerve agent GB, GF, VR, or VX. Peripheral tissues (diaphragm, heart, and skeletal muscle) and blood (red blood cell [RBC] and whole blood [WB]) were collected 60 min later.

†Control AChE activity (top row) was expressed as μmol substrate hydrolyzed/g protein/min for tissue samples and as μmol substrate hydrolyzed/ml/min for blood samples on the day of experiment. The AChE activity in nerve agent-exposed groups (bottom rows) was expressed as percentage of saline-treated control group. Values shown are mean ± S.E.M. with a group size of $n = 37$ for Control, 18 for GB, 7 for GF, 10 for VR, and 12 for VX, respectively.

Results

Signs of toxicity and lethality

Under the conditions of this study (without atropine therapy), 60 min following a 1.0 LD_{50} dose of GB, GF, VR, or VX all guinea pigs exhibited signs of intoxication, such as salivation, rhinorrhea, tremors, muscle fasciculations, and convulsions. In the case of GB and GF exposure, three of 21 (14%) and 20 of 27 (74%) animals, respectively, that were treated with saline died within 60 min (Table 3). One of nine animals (11%) exposed to GF and treated with SAD128 died during this period. One of eight (13%) and 12 of 19 (63%) animals exposed to GB and GF, respectively, and treated with ICD 4157, died. In the case of VR and VX exposure, all animals survived the 60 min after exposure.

AChE activity in brain regions

Basal AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum are shown in Table 2 (top row). The basal AChE activity of each brain region was significantly different from that in all other brain regions, peripheral tissues, and blood. The AChE activity in the striatum (389.3 ± 9.2 μmol substrate hydrolyzed/g protein/min) was the highest of any region. The cortex had the lowest AChE activity (56.9 ± 1.4 μmol substrate hydrolyzed/g protein/min) among all brain regions, which is about one seventh of that of the striatum. The rank order of basal AChE activity from high to low was striatum > cerebellum > brain stem > spinal cord > midbrain > hippocampus > cortex.

Effects of nerve agents

The ability of GB, GF, VR, and VX to inhibit brain regional AChE activity is shown in Table 2 (bottom rows). GB inhibition of AChE activity was significantly greater than that by all other agents tested in each brain region with two

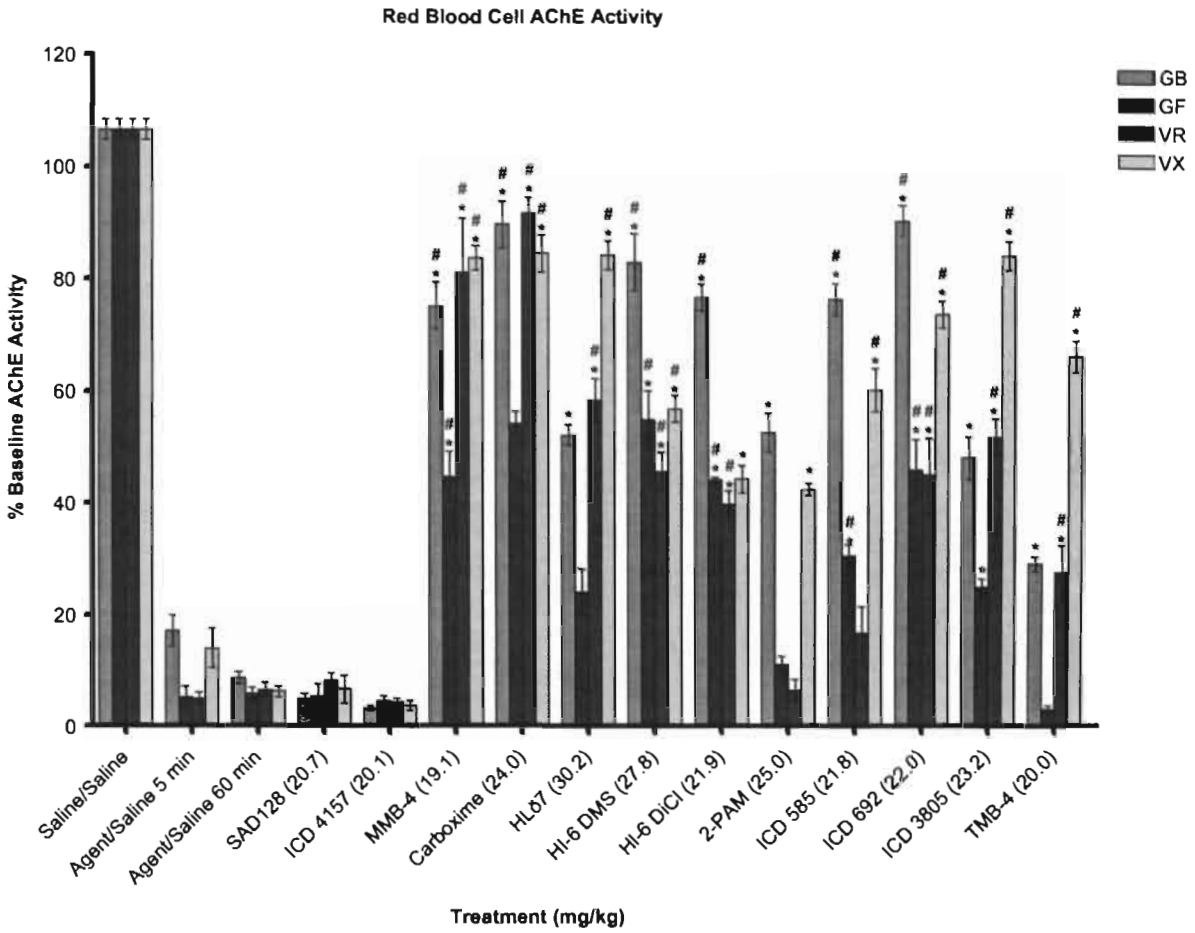

Figure 2. Effects of nerve agent and oxime treatments on red blood cell (RBC) AChE activity in the guinea pig. Saline, SAD128, ICD 4157, MMB-4, carboxime, HLö7, HI-6 DMS, HI-6 DiCl, 2-PAM, ICD 585, ICD 692, ICD 3805, or TMB-4 was given intramuscularly 5 min after a 1.0 LD_{50} subcutaneous dose of GB, GF, VR, or VX. The non-oximes (SAD128 and ICD 4157) served as negative controls of enzyme reactivation. Samples were collected at 60 min after nerve agent administration. AChE activity in RBC was expressed as percentage of individual baseline AChE activity. The baseline AChE activity was obtained 1–3 days prior to experimental treatment. Total AChE activity was expressed as mean ± SEM (% of control group) with 6–8 animals per group. 'Agent/Saline 5 min' data were taken from Shih et al. (2005). * Significantly different from nerve agent at 60 min, $p < 0.05$. # Significantly different from 2-PAM, $p < 0.05$. (See colour version of this figure online at www.informahealthcare.com/txm).

exceptions. In the hippocampus GB-inhibited AChE activity (78% inhibition) was significantly greater than GF-inhibited (62% inhibition) and VR-inhibited (64% inhibition) but not VX-inhibited (76% inhibition). In the striatum GB inhibited AChE activity (78% inhibition) significantly more than did VR (38% inhibition) and VX (42% inhibition) but not GF (67% inhibition). There was no difference in GF or VX inhibition of AChE activity in each brain region, except in the striatum where GF inhibited AChE activity (67% inhibition) significantly greater than did VX (42% inhibition). VR inhibition of AChE activity was significantly lower than that of all other agents tested in the brainstem (46% inhibition), cerebellum (59% inhibition), and spinal cord (29% inhibition). However, inhibition of AChE by VR was not significantly different from GF or VX inhibition in the cortex and hippocampus or VX inhibition in the midbrain or striatum.

Effects of oximes and non-oximes

At 60 min the AChE activities in any brain regions inhibited by a 1.0 LD_{50} dose of any nerve agent were not affected by the oxime treatments, with exceptions in two distinct instances. In the cortex, treatment with HLö7 significantly elevated the AChE activity inhibited by VR from 19.4 ± 1.8% (mean ± SEM) to 26.6 ± 2.5% of control. In the spinal cord, treatment with carboxime significantly elevated the AChE activity inhibited by VX from 46.4 ± 3.2% to 55.2 ± 2.4% of control.

The non-oximes (SAD128 and ICD 4157) did not affect or modify the AChE activities inhibited by any nerve agent in any brain regions.

AChE activity in peripheral tissues

Basal AChE activities in the diaphragm, heart and skeletal muscle (14.3 ± 0.8, 18.2 ± 0.6, and 10.5 ± 0.4 μmol substrate hydrolyzed/g protein/min, respectively) are shown in Table 1 (top). There was no significant difference among basal AChE activity in the peripheral tissues sampled.

Effects of nerve agents and non-oximes

The ability of GB, GF, VR, and VX to inhibit AChE activity in peripheral tissues is shown in Table 1 (bottom rows).

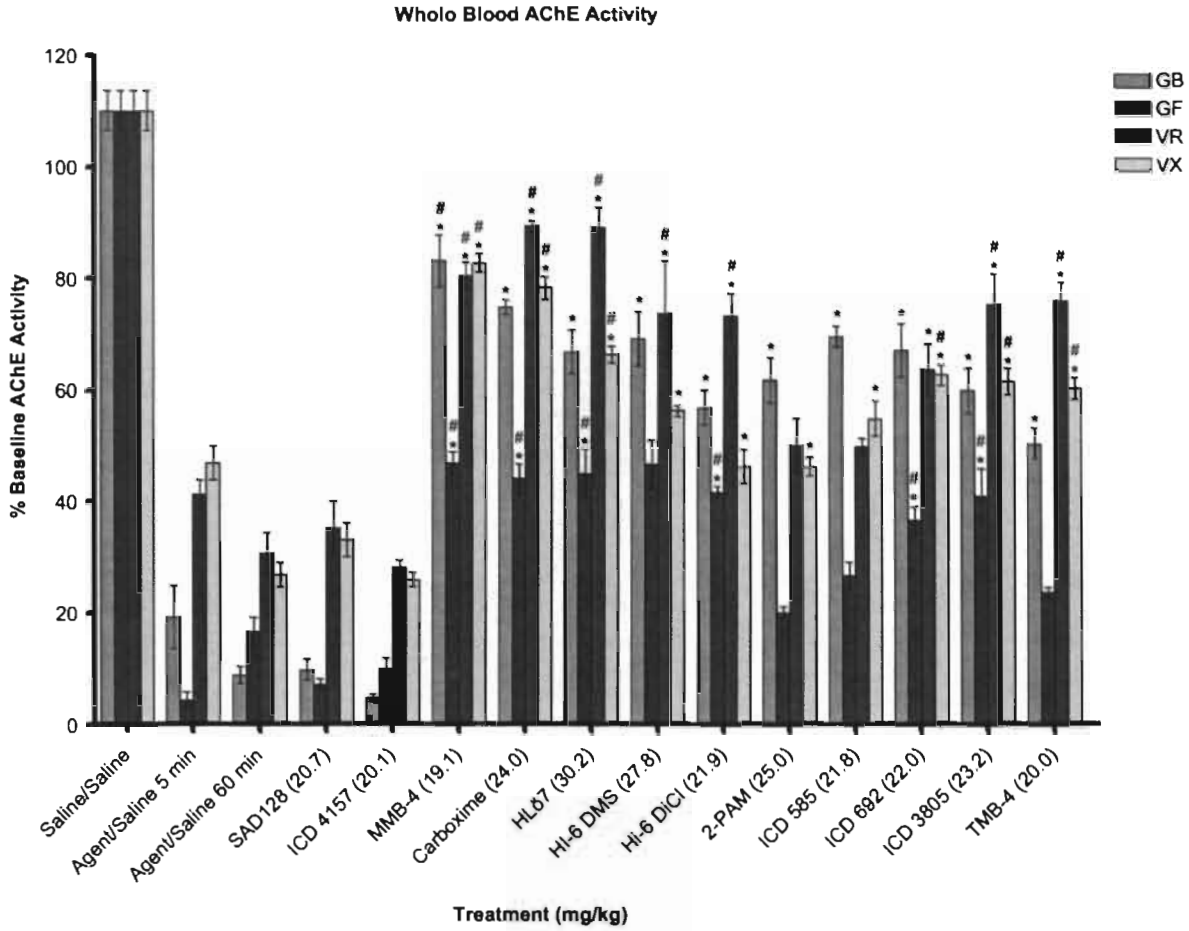

Figure 3. Effects of nerve agent and oxime treatments on whole blood (WB) AChE activity in the guinea pig. Saline, SAD128, ICD 4157, MMB-4, carboxime, HLö7, HI-6 DMS, HI-6 DiCl, 2-PAM, ICD 585, ICD 692, ICD 3805, or TMB-4 was given intramuscularly 5 min after a 1.0 LD_{50} subcutaneous dose of GB, GF, VR, or VX. The non-oximes (SAD128 and ICD 4157) served as negative controls of enzyme reactivation. Samples were collected at 60 min after nerve agent administration. AChE activity in WB was expressed as percentage of individual baseline AChE activity. The baseline AChE activity was obtained 1-3 days prior to experimental treatment. Total AChE activity was expressed as mean ± SEM (% of control group) with 6-8 animals per group. 'Agent/Saline 5 min' data were taken from Shih et al. (2005). * Significantly different from nerve agent at 60 min, $p<0.05$. # Significantly different from 2-PAM, $p<0.05$. (See colour version of this figure online at www.informahealthcare.com/txm).

Table 2. AChE activity in brain regions and spinal cord in control and nerve agent-intoxicated guinea pigs.*

	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain	Spinal cord	Striatum
Control AChE activity (μmol substrate hydrolyzed/g protein/min),# mean ± SEM							
Control	211.45 ± 6.99	234.66 ± 6.38	56.91 ± 1.42	99.20 ± 1.90	159.79 ± 4.23	189.59 ± 7.98	389.27 ± 9.15
% of control AChE activity at 60 min,# mean ± SEM							
GB	17.7 ± 2.4	10.7 ± 1.6	9.6 ± 1.2	22.0 ± 2.2	14.0 ± 1.5	27.3 ± 4.1	21.8 ± 2.3
GF	35.8 ± 4.9	23.5 ± 5.2	16.4 ± 2.7	38.2 ± 7.1	26.1 ± 3.7	47.6 ± 7.1	32.8 ± 6.3
VR	54.31 ± 2.9	40.8 ± 4.1	19.4 ± 1.8	36.5 ± 4.9	38.1 ± 2.9	71.42 ± 3.5	62.3 ± 3.8
VX	39.7 ± 2.0	27.3 ± 2.4	14.9 ± 1.2	23.6 ± 2.4	30.2 ± 1.8	46.4 ± 3.2	58.0 ± 2.8

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 LD_{50} dose of the nerve agent GB, GF, VR, or VX. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum) were collected 60 min later.

#The AChE activity in nerve agent-exposed groups was expressed as percentage of saline-treated control group. Values shown are mean ± S.E.M. with a group size of $n = 37$ for Control, 18 for GB, 7 for GF, 10 for VR, and 12 for VX, respectively.

Sixty minutes following a 1.0 LD_{50} dose of these nerve agents, in the diaphragm, GB and VX produced a similar degree of AChE inhibition (in the range of 72-74% inhibition), which was significantly higher than that induced by GF (65% inhibition) and by VR (52% inhibition). In the heart, the four nerve agents produced AChE inhibition that was significantly different among them. The rank order of AChE inhibition from high to low was GB > VX > GF > VR. In the skeletal muscle, similar to what was observed in the diaphragm, GB and VX produced similar AChE inhibition (in the range 66-68% inhibition), which was significantly greater than was produced by GF (52% inhibition) and by

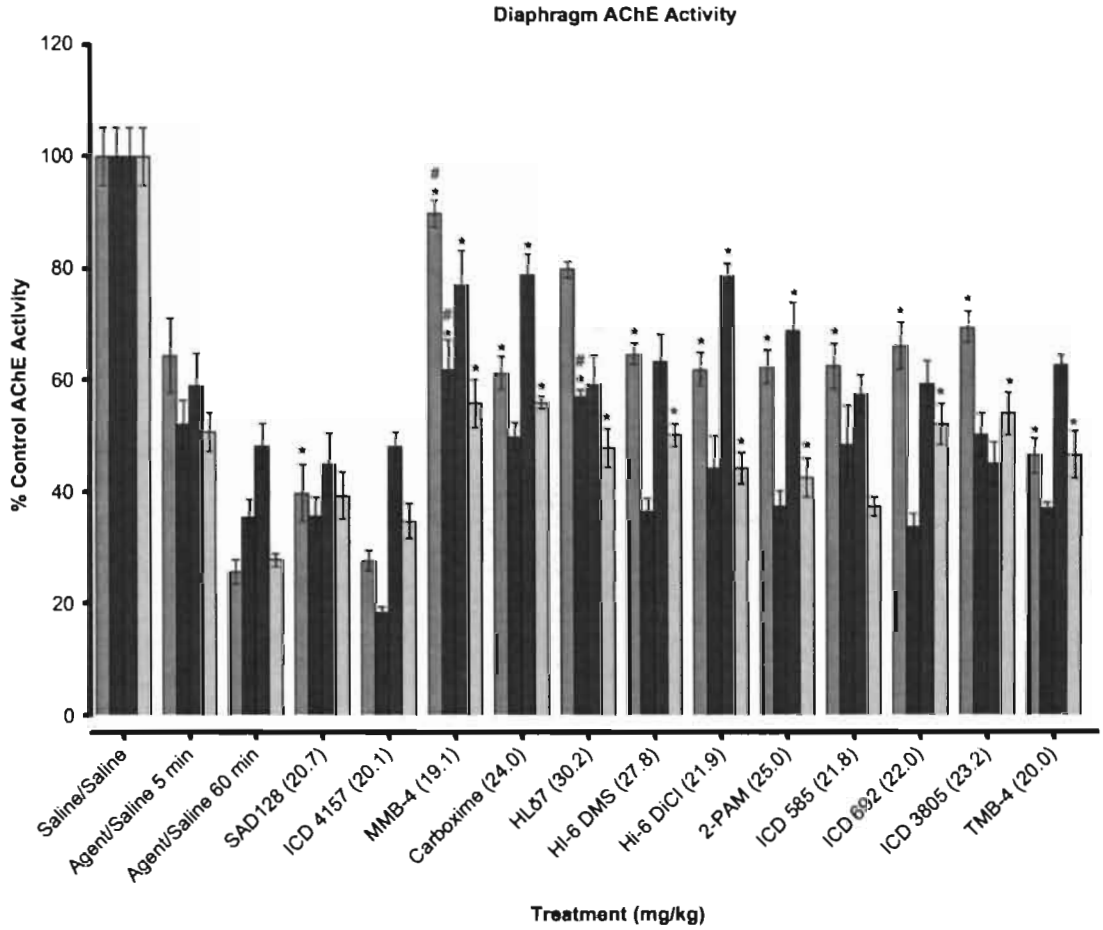

Figure 4. Effects of nerve agent and oxime treatments on diaphragm AChE activity in the guinea pig. Saline, SAD128, ICD 4157, MMB-4, carboxime, HLö7, HI-6 DMS, HI-6 DiCl, 2-PAM, ICD 585, ICD 692, ICD 3805, or TMB-4 was given intramuscularly 5 min after a 1.0 LD_{50} subcutaneous dose of GB, GF, VR, or VX. The non-oximes (SAD128 and ICD 4157) served as negative controls of enzyme reactivation. Samples were collected at 60 min after nerve agent administration. Total AChE activity was expressed as mean ± SEM (% of control group) with 6–8 animals per group. 'Agent/Saline 5 min' data were taken from Shih et al. (2005). * Significantly different from nerve agent at 60 min, $p < 0.05$. # Significantly different from 2-PAM, $p < 0.05$. (See colour version of this figure online at www.informahealthcare.com/txm).

VR (39% inhibition). Overall, GB and VX produced the most AChE inhibition and VR induced the least AChE inhibition among the four nerve agents in these three peripheral tissues.

The non-oximes (SAD128 and ICD 4157) did not modify the AChE activities inhibited by any nerve agent in these peripheral tissues, with two exceptions following GB exposure. In the diaphragm and skeletal muscle, the AChE activities showed an elevation from 25.7 ± 2.2% to 39.8 ± 5.0% of the control value and from 34.2 ± 3.6% to 57.4 ± 8.2% of the control value, respectively, in animals exposed to GB and treated with SAD128.

Effects of oximes

In the diaphragm (Figure 4), all oxime compounds studied were capable of reactivating AChE activity inhibited by a 1.0 LD_{50} dose of GB (25.7 ± 2.2% of control value). MMB-4 and HLö7 showed the highest levels of reactivation of diaphragm AChE, returning activity to 90.1 ± 2.4% and 80.1 ± 1.4%, respectively, of the control value. TMB-4 showed the least amount of reactivation in GB-inhibited diaphragm AChE, with activity reaching only 46.4 ± 3.1% of the control value. MMB-4 (90.1% of control value) and HLö7 (80.1% of control value) reactivated GB-inhibited diaphragm AChE to a significantly greater extent than did 2-PAM (62.5% control value).

MMB-4 and HLö7 were the only two oximes tested that reactivated diaphragm AChE activity inhibited by a 1.0 LD_{50} dose of GF (35.3 ± 3.3% of control value). MMB-4 showed the greatest level of reactivation following GF exposure, with activity of diaphragm AChE returning to 62.0 ± 5.4% of the control value. Both MMB-4 (62.0% of control value) and HLö7 (57.2% of control value) reactivated GF-inhibited diaphragm AChE significantly more than did 2-PAM (37.3% of control value).

MMB-4, carboxime, HI-6 DiCl, and 2-PAM were the only four oximes tested that reactivated diaphragm AChE inhibited by a 1.0 LD_{50} dose of VR (48.1 ± 4.1% of control value). Carboxime, HI-6 DiCl and MMB-4 displayed the highest levels of diaphragm AChE reactivation following VR exposure, returning AChE activity to 79.0 ± 3.8%, 78.8 ± 2.3%, and 77.1 ± 6.2%, respectively, of the control value. 2-PAM showed the least amount of reactivation by these oximes in

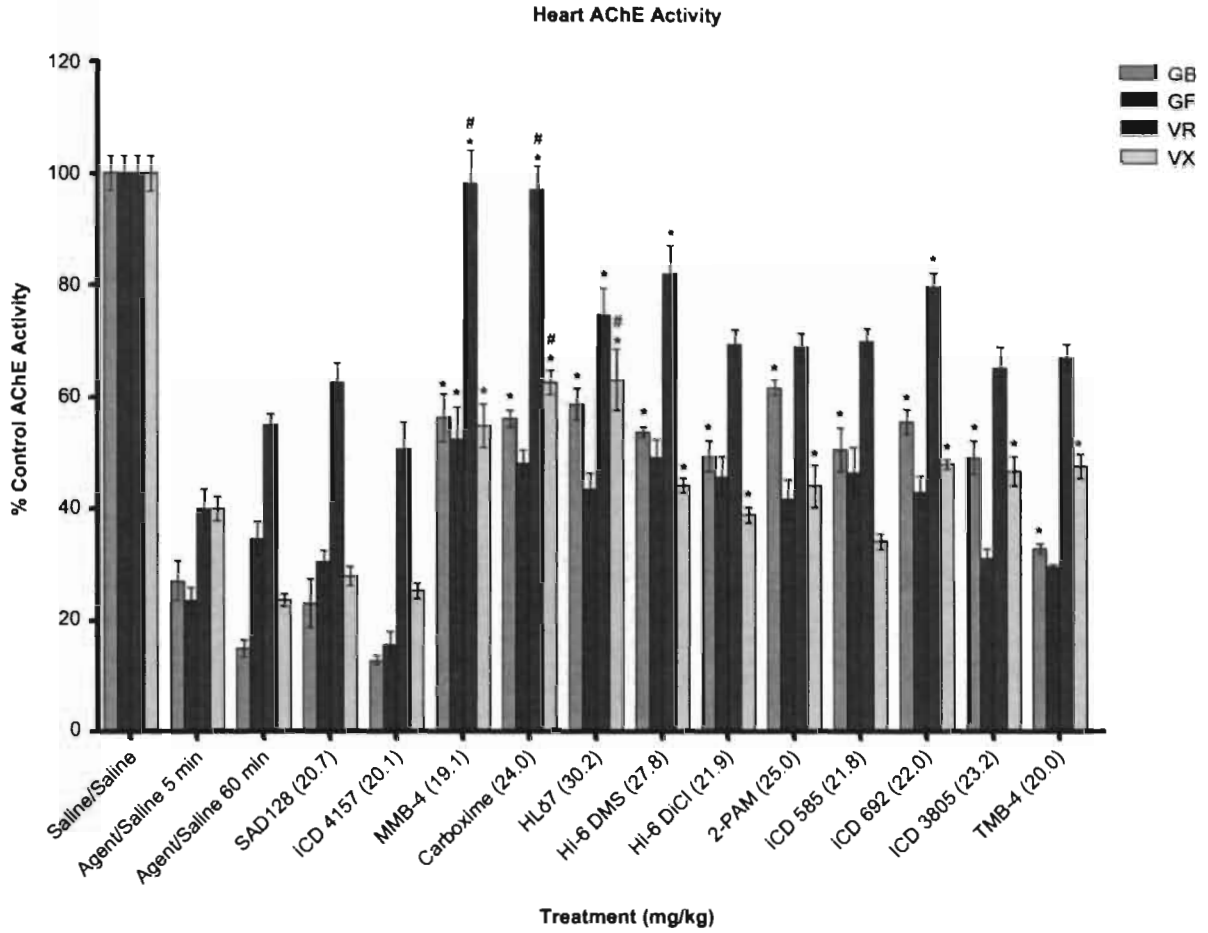

Figure 5. Effects of nerve agent and oxime treatments on heart AChE activity in the guinea pig. Saline, SAD128, ICD 4157, MMB-4, carboxime, HLö7, HI-6 DMS, HI-6 DiCl, 2-PAM, ICD 585, ICD 692, ICD 3805, or TMB-4 was given intramuscularly 5 min after a 1.0 LD_{50} subcutaneous dose of GB, GF, VR, or VX. The non-oximes (SAD128 and ICD 4157) served as negative controls of enzyme reactivation. Samples were collected at 60 min after nerve agent administration. Total AChE activity was expressed as mean ± SEM (% of control group) with 6–8 animals per group. 'Agent/Saline 5 min' data were taken from Shih et al. (2005). * Significantly different from nerve agent at 60 min, $p < 0.05$. # Significantly different from 2-PAM, $p < 0.05$. (See colour version of this figure online at www.informahealthcare.com/txm).

VR-inhibited diaphragm AChE, with activity reaching only 69.0 ± 5.0% of the control value.

Following VX exposure, all oxime compounds except ICD 585 were capable of reactivating inhibited diaphragm AChE (27.7 ± 1.2% of control value). MMB-4 showed the greatest level of reactivation following VX exposure, with activity of VX-inhibited diaphragm AChE returning to 56.0 ± 4.3% of the control value. 2-PAM showed the least amount of reactivation by these oximes in VX-inhibited diaphragm AChE, with activity reaching only 42.6 ± 3.5% of the control value.

In the heart (Figure 5), all oxime compounds were capable of reactivating AChE inhibition following a 1.0 LD_{50} dose of GB (15.0 ± 1.5% of control value). 2-PAM showed the greatest level of reactivation following GB exposure, with activity of GB-inhibited heart AChE returning to 61.7 ± 1.5% of the control value. TMB-4 showed the least amount of reactivation in GB-inhibited heart AChE, with activity reaching 32.9 ± 0.9% of the control value.

Following GF exposure, MMB-4 was the only oxime able to reactivate inhibited heart AChE activities (from 34.7 ± 2.9% to 52.3 ± 5.6% of control value).

MMB-4, carboxime, HLö7, HI-6 DMS, and ICD692 were all capable of reactivating heart AChE inhibited by a 1.0 LD_{50} dose of VR (54.9 ± 2.0% of control value). MMB-4 and carboxime exhibited the highest levels of reactivation following VR exposure, returning heart AChE activity to 97.8 ± 6.0% and 96.7 ± 4.0%, respectively, of the control value. HLö7 showed the least amount of reactivation by these oximes in VR-inhibited heart AChE, where activity reached 74.6 ± 4.8% of the control value. MMB-4 (97.8 ± 6.0% of control value) and carboxime (96.9 ± 4.0% of control value) reactivated VR-inhibited heart AChE significantly more than did 2-PAM (68.9 ± 2.5% of control value).

ICD585 was the only oxime not capable of reactivating heart AChE activities inhibited by a 1.0 LD_{50} dose of VX (23.6 ± 1.1% of control value). HLö7 showed the greatest level of reactivation following VX exposure with activity of VX-inhibited heart AChE returning to 63.0 ± 5.6% of the control value. HI-6 DiCl showed the least amount of reactivation by these oximes in VX-inhibited heart AChE, with activity reaching 38.9 ± 1.4% of the control value. Carboxime (62.5% of control value) and HLö7 (63.0% of control value)

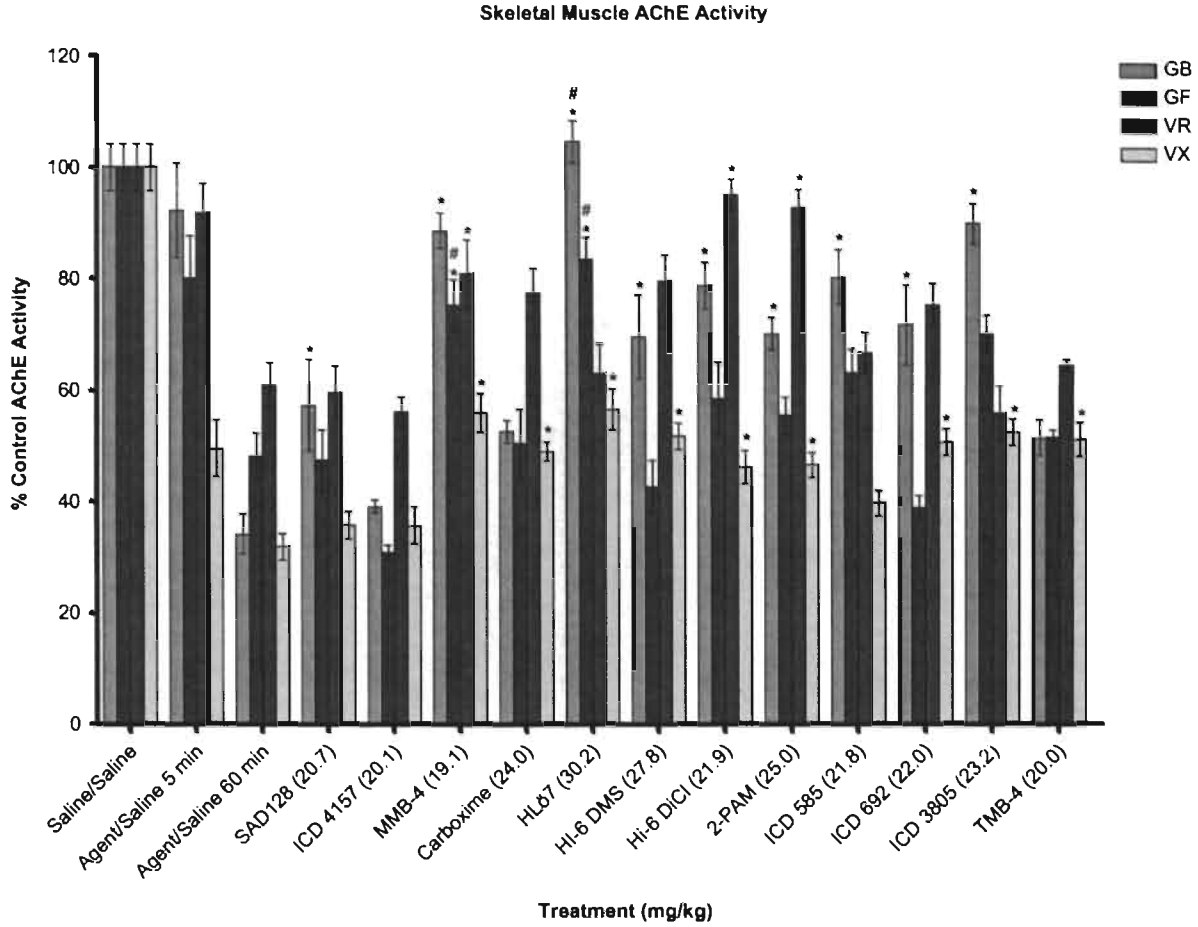

Figure 6. Effects of nerve agent and oxime treatments on skeletal muscle AChE activity in the guinea pig. Saline, SAD128, ICD 4157, MMB-4, carboxime, HLö7, HI-6 DMS, HI-6 DiCl, 2-PAM, ICD 585, ICD 692, ICD 3805, or TMB-4 was given intramuscularly 5 min after a 1.0 LD_{50} subcutaneous dose of GB, GF, VR, or VX. The non-oximes (SAD128 and ICD 4157) served as negative controls of enzyme reactivation. Samples were collected at 60 min after nerve agent administration. Total AChE activity was expressed as mean ± SEM (% of control group) with 6–8 animals per group. 'Agent/Saline 5 min' data were taken from Shih et al. (2005). * Significantly different from nerve agent at 60 min, $p < 0.05$. # Significantly different from 2-PAM, $p < 0.05$. (See colour version of this figure online at www.informahealthcare.com/txm).

Table 3. Mortality within 60 min after nerve agent exposure.*

Treatment	GB	GF	VR	VX
Saline/saline	0/8	0/11	0/10	0/8
NA/saline	3/21 (14%)	20/27 (74%)	0/10	0/12
NA/SAD128	0/8	1/9 (11%)	0/8	0/8
NA/ICD 4157	1/8 (13%)	12/19 (63%)	0/8	0/8

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 LD_{50} dose of the nerve agent (NA) GB, GF, VR, or VX and treated intramuscularly 5 min later with saline (0.5 ml/kg), SAD128 (20.7 mg/kg), or ICD 4157 (20.1 mg/kg). At 60 min after NA administration animals were taken for experimental procedure. Numerator = number of animals died; denominator = total animals tested.

reactivated VX-inhibited heart AChE significantly more than did 2-PAM (44.1% of control value).

In skeletal muscle (Figure 6), all oximes studied, with the exception of carboxime and TMB-4, were capable of reactivating AChE activity inhibited by a 1.0 LD_{50} dose of GB (34.2 ± 3.6% of control value). HLö7 showed the greatest level of reactivation following GB exposure, with activity of GB-inhibited skeletal muscle AChE reaching 104.8 ± 3.8% of the control value; ICD 3805 and MMB-4 also displayed an excellent level of reactivation, with GB-inhibited skeletal muscle AChE reaching 89.7 ± 3.6% and 88.7 ± 3.2%, respectively, of the control value. HLö7 reactivated GB-inhibited skeletal muscle AChE significantly more than did 2-PAM (70.2 ± 2.8% of control value). HI-6 DMS showed the least amount of reactivation among these oximes in GB-inhibited skeletal muscle AChE; activity reached 69.8 ± 7.4% of the control value.

MMB-4 and HLö7 were the only two oximes tested that reactivated skeletal muscle AChE activity inhibited by a 1.0 LD_{50} dose of GF (48.3 ± 4.1% of control value). MMB-4 and HLö7 both showed excellent levels of reactivation following GF exposure, with the activity of GF-inhibited skeletal muscle AChE returning to 75.5 ± 4.6% and 83.7 ± 3.8% of the control value, respectively.

MMB-4, HI-6 DiCl and 2-PAM were the only three oximes tested that reactivated skeletal muscle AChE activity inhibited by a 1.0 LD_{50} dose of VR (60.9 ± 4.0% of control value). HI-6 DiCl showed greater reactivation than did 2-PAM and MMB-4, returning activity of VR-inhibited skeletal

Table 4. Summary of the significant AChE reactivation by oxime treatments in diaphragm, heart, and skeletal muscle following exposure to GB, GF, VR, and VX.*

	MMB-4	HLö7	Carboxime	HI-6 DMS	HI-6 DiCl	2-PAM	ICD585	ICD692	ICD3805	TMB-4
GB	+++	+++	++	+++	+++	+++	+++	+++	+++	++
GF	+++	++	–	–	–	–	–	–	–	–
VR	+++	+	++	+	++	++	–	+	–	–
VX	+++	+++	+++	+++	+++	+++	–	+++	+++	+++

* For each of the peripheral tissue (diaphragm, heart, or skeletal muscle), an oxime treatment that significantly reactivated a nerve agent-inhibited AChE activity was assigned a '+' sign. Thus, if an oxime treatment significantly reactivated AChE in all three peripheral tissues following a nerve agent exposure, its cell received '+++' maximum. When an oxime treatment did not significantly reactivate a nerve agent-inhibited AChE in any tissue, it was assigned a '–' sign.

muscle AChE to 95.1%, 92.7%, and 81.1% of the control value, respectively.

All oximes except ICD 585 were capable of reactivating skeletal muscle AChE activity inhibited by a 1.0 LD_{50} dose of VX (32.1 ± 2.4% of control value). HLö7 showed the greatest level of reactivation following VX exposure, with activity of VX-inhibited skeletal muscle AChE returning to 56.7 ± 3.7% of the control value. HI-6 DiCl showed the least amount of reactivation by these oximes in VX-inhibited skeletal muscle AChE, with activity reaching 46.3 ± 3.0% of the control value.

A summary of the capability of oxime treatments to significantly reactivate the inhibited AChE activity in three peripheral tissues by the four nerve agents is shown in Table 4.

AChE activity in blood

Basal AChE activities in RBC and WB are shown in Table 1 (top row). The basal AChE activities in RBC and WB are 2.2 ± 0.06 and 2.5 ± 0.06 μmol substrate hydrolyzed/ml/min, respectively.

Effects of nerve agents and non-oximes

As can be seen from Table 1 (bottom rows), the AChE activities in RBC were markedly inhibited 60 min following GB, GF, VR, and VX (at a 1.0 LD_{50} dose), to 8.7 ± 1.1%, 5.9 ± 1.1%, 6.6 ± 1.2%, and 6.3 ± 1.0% of control value, respectively. Thus, all four nerve agents produced a similar degree of AChE inhibition.

In the WB, however, GB, GF, VR, and VX reduced enzyme activity to 8.8 ± 1.5%, 16.6 ± 2.7%, 30.8 ± 3.6%, and 26.8 ± 2.1% of control value, respectively. As can be seen, both GB and GF produced significantly greater AChE inhibition than did VR and VX. The latter two nerve agents showed a similar degree of AChE inhibition.

The non-oximes (SAD128 and ICD 4157) did not significantly modify the AChE activities inhibited by any nerve agent in any blood samples.

Effects of oximes

In RBC (Figure 2), all oximes studied were capable of significantly reactivating AChE activity inhibited by a 1.0 LD_{50} dose of GB (8.7 ± 1.1% of control value). MMB-4 (75% of control value), carboxime (90% of control value), HI-6 DMS (83% of control value), HI-6 DiCl (77% of control value), ICD 585 (76% of control value), and ICD 692 (90% of control value) were capable of reactivating GB-inhibited RBC AChE activity significantly more than was 2-PAM (53% of control value). Carboxime and ICD 692 showed the highest levels of reactivation following GB exposure, with activity of GB-inhibited RBC AChE returning to 89.8 ± 4.3% and 90.2 ± 2.7% of the control value, respectively. TMB-4 showed the least amount of reactivation in GB-inhibited RBC AChE, in that reactivated activity reached 29.0 ± 1.3% of the control value.

All oximes except 2-PAM and TMB-4 were capable of significantly reactivating RBC AChE activity inhibited by a 1.0 LD_{50} dose of GF (5.9 ± 1.1% of the control value). Carboxime and HI-6 DMS showed the highest levels of reactivation following GF exposure, returning GF-inhibited RBC AChE activity to 54.3 ± 2.1% and 55.0 ± 5.1% of the control value, respectively. HLö7 showed the least amount of reactivation among these oximes in GF-inhibited RBC AChE, with activity reaching 24.1 ± 4.1% of the control value.

All oximes except 2-PAM and ICD 585 were capable of significantly reactivating RBC AChE activity inhibited by a 1.0 LD_{50} dose of VR (6.6 ± 1.2% of the control value). MMB-4 and carboxime showed the highest levels of reactivation following VR exposure: activity of VR-inhibited RBC AChE returned to 81.2 ± 9.8% and 91.9 ± 2.8% of the control value, respectively. TMB-4 showed the least amount of reactivation by these oximes in VR-inhibited RBC AChE: reactivated activity reached 27.5 ± 4.7% of the control value.

All oximes tested were capable of significantly reactivating RBC AChE activity inhibited by a 1.0 LD_{50} dose of VX (6.3 ± 1.0% of the control value). MMB-4, carboxime, HLö7, and ICD 3805 showed excellent levels of reactivation following VX exposure, with activity of VX-inhibited RBC AChE returning to 83.9 ± 2.1%, 84.7 ± 3.4%, 84.4 ± 3.4%, 83.9 ± 2.7% of the control value, respectively. HI-6 DMS showed the least amount of reactivation by these oximes in VX-inhibited RBC AChE, with activity reaching 56.8 ± 2.3% of the control value.

In WB (Figure 3), all oximes studied were capable of significantly reactivating AChE activity inhibited by a 1.0 LD_{50} dose of GB (8.8 ± 1.5% of the control value). MMB-4 showed the greatest level of reactivation following GB exposure, with activity of GB-inhibited WB AChE returning to 83.1 ± 4.6% of the control value. MMB-4 (83.1% of control value) also reactivated GB-inhibited WB AChE activity significantly more than did 2-PAM (61.8% of control value). TMB-4 showed the least amount of reactivation in GB-inhibited WB AChE, with activity reaching 50.5 ± 2.7% of the control value.

All oximes except 2-PAM, ICD 585, and TMB-4 were capable of significantly reactivating WB AChE activity inhibited by a 1.0 LD_{50} dose of GF (16.6 ± 2.7% of the control value).

MMB-4 showed the greatest level of reactivation following GF exposure, with activity of GF-inhibited WB AChE returning to 47.0 ± 2.0% of the control value. ICD 692 showed the least amount of reactivation by these oximes in GF-inhibited WB AChE, with activity reaching 36.7 ± 2.4% of the control value.

All oximes except 2-PAM and ICD 585 were capable of significantly reactivating WB AChE activity inhibited by a 1.0 LD_{50} dose of VR (30.8 ± 3.6% of the control value). Carboxime and HLö7 showed the highest levels of reactivation following VR exposure such that activity of VR-inhibited WB AChE returned to 89.3 ± 0.8% and 89.1 ± 3.4% of the control value, respectively. ICD 692 showed the least amount of reactivation by these oximes in VR-inhibited WB AChE, returning activity to 63.7 ± 4.7% of the control value.

All oximes were capable of significantly reactivating WB AChE activity inhibited by a 1.0 LD_{50} dose of VX (26.8 ± 2.1% of the control value). MMB-4 and carboxime showed the highest levels of reactivation following VX exposure, with activity of VX-inhibited WB AChE returning to 82.7 ± 1.7% and 78.2 ± 2.1% of the control value, respectively. HI-6 DiCl showed the least amount of reactivation by these oximes in VX-inhibited WB AChE with a return in activity of 46.2 ± 3.0% of the control value.

A summary of the capability of oxime treatments to reactivate the inhibited AChE activity in RBC and WB by the four nerve agents is shown in Table 5.

Overall assessment of oxime reactivating capacity

We divided the analysis of AChE reactivation data into two compartments. First of all, Table 4 shows a summary of the statistically significant AChE reactivation by oxime treatments in peripheral tissues (diaphragm, heart, and skeletal muscle) following exposure to GB, GF, VR, and VX. For each of the peripheral tissues, an oxime treatment that significantly reactivated nerve agent-inhibited AChE activity was assigned a '+' sign. Thus, if an oxime treatment significantly reactivated AChE in all three peripheral tissues following a nerve agent exposure, it received '+++' maximum. A '–' sign denoted an oxime treatment that did not significantly reactivate a nerve agent-inhibited AChE in any tissue. It is striking to notice that MMB-4 is the only oxime capable of reactivating AChE inhibited by all four nerve agents in all three peripheral tissues. HLö7 was also capable of reactivating AChE inhibited by these four nerve agents, but with a weaker action toward VR- and GF-inhibited tissue AChE activity. The reactivating potency of carboxime, HI-6 DMS, HI-6 DiCl, 2-PAM, and ICD 692 appears to be equal. They all lacked the ability to reactivate GF-inhibited AChE in the peripheral tissues. The DiCl and DMS salts of HI-6 exhibited similar potency against GB, VR, and VX. Among them, ICD 585, which was not able to reactivate peripheral tissue AChE activity inhibited by GF, VR, or VX, was the worst oxime reactivator. This table also showed that the relative AChE reactivating potency of these oxime compounds against the four nerve agents was GB > VX > VR > GF.

Second, Table 5 shows a summary of the statistically significant AChE reactivation by oxime treatments in the blood components (RBC and WB) following exposure to GB, GF, VR, and VX. For each of the blood components, an oxime treatment that significantly reactivated nerve agent-inhibited AChE activity was assigned a '+' sign. Thus, if an oxime treatment significantly reactivated AChE in both RBC and WB following a nerve agent exposure, it received '++' maximum. A '–' sign denoted an oxime treatment that did not significantly reactivate nerve agent-inhibited AChE in any blood component. All oxime compounds appeared to be able to reactivate AChE inhibited by the four nerve agents in blood components, with several exceptions: TMB-4 was not capable of reactivating GF-inhibited AChE in RBC and WB; ICD 585 was not able to reactivate VR-inhibited AChE in RBC and WB, nor GF-inhibited AChE in WB; and 2-PAM was not capable of reactivating GF- and VR-inhibited AChE in either RBC or WB.

Discussion

It has generally been recognized that the acute toxic manifestations of exposure to OP nerve agents are due to the irreversible binding of the agents to the ChE class of enzymes, in particular to AChE, which serves to hydrolyze and degrade the released cholinergic neurotransmitter ACh at the synaptic junction of the central and peripheral cholinergic nervous systems (Taylor, 2001). Excess ACh results in uncontrolled stimulation followed by blockade of neuronal transmission. Reactivation of inhibited AChE was identified as a beneficial pharmacologic approach, leading to the use of 2-PAM and obidoxime (Toxogonin®) for treatment of OP poisoning over 50 years ago (Childs et al., 1955; Wilson and Ginsburg, 1955; Hobinger and Sadler, 1959). Even though several oxime reactivators such as 2-PAM, P2S, obidoxime, TMB-4, MMB-4, or HI-6 have been clinically used for therapy of OP poisoning in many countries around the world, the only licensed oxime in the US for the treatment of nerve agent exposure

Table 5. Summary of the significant AChE reactivation by oxime treatments in red blood cell (RBC) and whole blood (WB) following exposure to GB, GF, VR, and VX.*

	MMB-4	HLö7	Carboxime	HI-6 DMS	HI-6 DiCl	2-PAM	ICD585	ICD692	ICD3805	TMB-4
GB	++	++	++	++	++	++	++	++	++	++
GF	++	++	++	++	++	–	+	++	++	–
VR	++	++	++	++	++	–	–	++	++	++
VX	++	++	++	++	++	++	++	++	++	++

* For each of the blood components (RBC or WB), an oxime treatment that significantly reactivated a nerve agent-inhibited AChE activity was assigned a '+' sign. Thus, if an oxime treatment significantly reactivated AChE in both RBC and WB following a nerve agent exposure, its cell received '++' maximum. When an oxime treatment did not significantly reactivate a nerve agent-inhibited AChE in any blood sample, it was assigned a '–' sign.

is 2-PAM (Moore et al., 1995; Aas, 2003). While 2-PAM has acceptable efficacy against certain nerve agents (e.g. GB, VX), it lacks the desired level of efficacy against other nerve agents (e.g. tabun, soman, GF, VR), even when combined with PB pre-treatment and atropine sulfate and diazepam treatment (Boskovic et al., 1984). Thus, there is a need to identify and develop AChE reactivators with a broader spectrum of efficacy. In recent years, several oximes, such as HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD 585, ICD 692, and ICD 3805, have been found to possess much better antidotal capacity than 2-PAM does in response to intoxication to OP pesticides or several nerve agents in animal studies (Dawson, 1994; Kassa, 1998; 2002; 2005; Nechiporenko and Zatsepin, 2003; Dishovsky, 2005; Kokshareva et al., 2005; Antonijevic and Stojiljkovic, 2007; Eyer, 2008; I. Koplovitz, unpublished observations). Even though several of these oximes were studied for their ability to reactivate VX-, GF-, or soman-inhibited AChE activity individually in vivo (Harris and Stitcher, 1983; Lundy and Shih, 1983; Harris et al., 1989; 1990; Shih et al., 1991; Shih, 1993; Koplovitz and Stewart, 1994; Kassa and Cabal, 1999), their capacity to reactivate AChE activity inhibited by various OP nerve agents had not been evaluated and compared systematically. Therefore, in the present study we compared these oximes with 2-PAM in their ability to reactivate inhibited AChE in blood and tissues with the goal to identify a broader spectrum oxime that is significantly more potent and effective than 2-PAM as an antidote for numerous OP nerve agents.

In this study, we purposely did not conduct experimental exposures with the OP nerve agents tabun and soman for the following reasons. Oxime moieties can restore inactivated AChE by nucleophilic displacement of the OP moiety from the active-site serine, provided that they can access the phosphoserine linkage. The chemical structure of tabun with its amide nitrogen makes this particularly problematic. The relative inability of oximes to restore tabun-inhibited AChE has been attributed, at least in part, to nuclophilic impedance (Ekstrom et al., 2006a; b; Hoskovcova et al., 2007). Other AChE-inhibitors, such as soman, become refractory to reactivation by an 'aging' process (Fleisher and Harris, 1965; Fleisher et al., 1967). At some point after inactivation, nerve agents can undergo dealkylation, resulting in the formation of negatively charged methylphosphonate AChE, which is held to present an electrostatic barrier to nucleophilic attack by oximes. This, combined with structural shifts in this OP-AChE complex, renders the AChE-OP complex irreversible. Soman aging is extremely rapid, with a half life of 1–8 min depending on species (Talbot et al., 1988; Luo et al., 2007). At the time of oxime administration in this experiment, the OP-AChE complex would likely be irreversible in an animal exposed to soman. In contrast, the half-life of reactivatable OP-AChE exceeds 3 h for GB and is even longer for GF and VX (Worek et al., 2004; Luo et al., 2007). All the oximes studied (i.e. 2-PAM, HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD 585, ICD 692, and ICD 3805) proved good reactivators of AChE inhibited by GB, GF, VR, and VX to different degrees. Their actions, in general, were limited to blood and peripheral tissues. This supports the observations made by many laboratories that the quaternary mono- or bis-pyridinium oxime compounds do not readily reactivate nerve agent-inhibited AChE in the central nervous system, even though there was controversy as to the possible entrance of these oximes into the brain. In the present study, reactivation was only observed in the cortex where treatment with HLö7 significantly elevated the AChE activity inhibited by VR by a 7% margin. Carboxime has also been described as able to reactivate ChE inhibited by GB and VX in rat brain (Nechiporenko and Zatsepin, 2003; Kokshareva et al., 2005). However, we observed this action only in the spinal cord where treatment with carboxime significantly elevated the AChE activity inhibited by VX by a 9% increase above the agent-inhibited enzyme activity. The physiological significance of these modest levels of reactivation in these two cases is not immediately clear.

As was reported earlier OP nerve agents produced tissue compartment specificity in their ability to inhibit AChE activity (Shih et al., 2005); the oximes studied here also exhibited differential potency in reactivating nerve agent-inhibited tissue AChE activity. When examined carefully the results obtained from the present study showed that MMB-4 was the only oxime (among oxime compounds tested) capable of reactivating AChE inhibited by all four nerve agents in the blood components and all three peripheral tissues (Tables 4 and 5). Additionally, in several cases, AChE activities in animals treated with MMB-4 reached control values: in diaphragm and skeletal muscle after GB and in heart after VR exposures. HLö7 was second in its overall reactivating capacity. In RBC and WB it reactivated AChE inhibited by all four nerve agents. Skeletal muscle AChE activities in animals treated with HLö7 after GB exposure returned to control value. Although HLö7 was capable of reactivating AChE in all three peripheral tissues inhibited by GB and VX, it lacked the ability to reactivate AChE inhibited in the diaphragm and skeletal muscle by VR and AChE inhibited in the heart by GF. Carboxime reactivated AChE in RBC and WB inhibited by all four nerve agents and was capable of reactivating VX-inhibited AChE in all three and GB-inhibited AChE in two peripheral tissues. It returned the AChE activity in the heart to the control value after VR exposure. However, carboxime was not able to reactivate GF-inhibited AChE in diaphragm, heart, and skeletal muscle, and VR-inhibited AChE in skeletal muscle. HI-6 showed a complete nerve agent preference. It was capable of reactivating GB-, VR-, and VX-inhibited AChE, but not GF-inhibited AChE, in blood or tissue. There was no difference in AChE reactivating capacity between the two salts of HI-6 following exposure to the four nerve agents studied (Krummer et al., 2002). Similarly, TMB-4 was not capable of reactivating GF-inhibited AChE in blood and peripheral tissues. It lacked the ability to reactivate GB-inhibited AChE in the skeletal muscle and VR-inhibited enzyme activity in the diaphragm, heart, and skeletal muscle. Although 2-PAM was not able to reactivate GF-inhibited AChE in blood and any peripheral tissue tested, it was an excellent

reactivator of AChE inhibited by GB and VX in blood, and in all three peripheral tissues. It was also not able to reactivate VR-inhibited AChE in RBC, WB, and the heart. On the other hand, although the other three ICD compounds (ICD 585, ICD 692, and ICD 3805) were, in general, able to reactivate AChE in the blood inhibited by all four nerve agents, these compounds lacked the capability to do so on GF- and VR-inhibited AChE in diaphragm, heart, and skeletal muscle. Overall, ICD 585 was not able to reactivate GF-, VR-, and VX-inhibited AChE, but was able to reactivate GB-inhibited AChE in all three peripheral tissues.

The present results also showed that these oximes were able to reactive AChE inhibited by four different nerve agents in peripheral tissues to different degrees (Table 4). The rank order of susceptibility for in vivo AChE reactivation by oxime treatments in peripheral tissues was GB > VX > VR > GF.

It is interesting to note that the capability of an oxime treatment to reactivate AChE in the blood and the peripheral tissue compartments was markedly different. It was understandable that the oxime compound entered the blood first before reacting in the tissue compartment and, thus, it had a better chance to act on the AChE inhibited in the blood (i.e. proximity effect). Therefore, it was observed that most of the oximes were capable of reactivating to a greater degree the nerve agent-inhibited AChE in the blood than that in the peripheral tissues. For example, with the exception of 2-PAM and TMB-4, all oximes tested displayed significant AChE reactivation in the blood components, while in the peripheral tissue component only MMB-4 and HLö7 were capable of doing so. Furthermore, the majority of the oximes studied were capable of reactivating AChE inhibited by the four nerve agents in the blood components significantly better than was 2-PAM (Figures 2 and 3). In the peripheral tissue compartment, however, only MMB-4, HLö7, or carboxime displayed significantly better AChE reactivating capacity than did 2-PAM, but only in a few tissues (Figures 4–6). There was no relation between blood and peripheral tissues in the reactivating efficacy of oxime treatments. Therefore, a higher blood AChE activity following oxime treatment in nerve agent poisoning could not be used as a direct indicator of elevated AChE activity in a peripheral tissue (diaphragm, heart, or skeletal muscle).

In the present study, we used two structurally similar non-oxime compounds, namely SAD128 and ICD 4157, as negative controls for AChE reactivation analysis. It is interesting to note some differences between the actions of these two compounds. ICD 4157 acted just like a saline control treatment; it neither reactivated nerve agent-inhibited AChE in blood and peripheral tissues, nor affected either GB- or GF-induced mortality within 60 min after agent exposure when compared with nerve agent/saline treatment (Table 3). In contrast, SAD128 treatment prevented mortality when compared with the nerve agent/saline treatment (Table 3; 0% and 11% for GB and GF exposure, respectively). It also had significantly elevated AChE activity in diaphragm and in skeletal muscle of GB-exposed animals when compared with the GB/saline treatment group. This finding confirmed the observations made for SAD128 by Clement and Erhardt (1994), who reported in an in vitro study that SAD128 treatment 5 min after exposure to the nerve agent soman resulted in significantly greater total AChE activity in rat diaphragm tissue. There have been many speculations on the beneficial effects of SAD128, for example shielding of AChE from inhibition by soman (Harris et al., 1978; Stalc and Sentjurc, 1990), blockage of the muscarinic receptors (Amitai et al., 1980), or blockage of the nicotinic receptor ion channel (Tattersall, 1993). However, we observed this phenomenon only in the case of GB-inhibited tissue AChE. The reason for this is not clear.

In summary, the present results showed that the OP nerve agents GB, GF, VR, and VX caused different degrees of AChE inhibition in various tissue compartments. The oximes also showed differential potency in reactivating nerve agent-inhibited AChE in various peripheral tissues and exhibited differential AChE reactivating specificity for nerve agents. Reactivation of nerve agent-inhibited AChE in the brain or spinal cord was not clearly demonstrated. AChE inhibited by GB was most susceptible and those inhibited by GF was the least susceptible to oxime reactivation. There was no difference in the AChE-reactivating potency of the DiCl and DMS salts of HI-6 in any blood or peripheral tissue compartment. Overall, MMB-4 appeared to be the broadest spectrum in vivo reactivator for GB, GF, VX, and VR intoxication. The relationship between the AChE reactivation observed with these oximes in peripheral tissues and the survival following exposure to these nerve agents is currently under investigation.

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